Epilepsia, **45**(7):864–867, 2004
Blackwell Publishing, Inc.
© 2004 International League Against Epilepsy

Brief Communication

Allopregnanolone Analogs That Positively Modulate $GABA_A$ Receptors Protect against Partial Seizures Induced by 6-Hz Electrical Stimulation in Mice

Rafal M. Kaminski, Matthew R. Livingood, and Michael A. Rogawski

Epilepsy Research Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland, U.S.A.

Summary: *Purpose:* Low-frequency (6 Hz), long-duration (3 s) electrical stimulation in mice produces seizures characterized by immobility, focal clonus, and automatic behaviors reminiscent of human limbic epilepsy. Renewed interest has been expressed in this seizure model with the recognition that it is sensitive to a broad spectrum of anticonvulsants (AEDs) and may have distinct pharmacologic responsiveness from other in vivo tests of AED efficacy. Here we sought to determine whether the progesterone-derived neuroactive steroid allopregnanolone (5α,3α-P) and several structural analogues with varying degrees of activity as positive allosteric modulators of γ-aminobutyric acid $(GABA)_A$ receptors are protective in the 6-Hz seizure model.

Methods: Mice were pretreated with neuroactive steroids (15 min before) or clonazepam (CZP; 30 min before) to 6-Hz corneal stimulation (32 mA). Animals that failed to exhibit immobility were considered protected.

Results: The neuroactive steroids prevented 6-Hz seizures with rank order of potencies (ED_{50} values): ganaxolone (6.3 mg/kg) > 5α,3α-P (14.2 mg/kg) $\geq$ 5β,3α-P (14.4 mg/kg) > 5α,3β-P (>100 mg/kg). CZP also was protective (ED_{50} value, 0.075 mg/kg). The potencies of the neuroactive steroids and CZP are similar to their previously reported activities in the pentylenetetrazol (PTZ) seizure model.

Conclusions: Neuroactive steroids have comparable potencies in the 6-Hz and PTZ models. Their structural specificity in both models corresponds with their activities as positive allosteric modulators of $GABA_A$ receptors, although ganaxolone is more potent than expected, probably because it has greater bioavailability. The 6-Hz model may be a valuable tool in drug development for the identification of GABAergic AEDs. **Key Words:** Neuroactive steroid—Ganaxolone—Allopregnanolone—Clonazepam—$GABA_A$-receptor modulation—Seizure—6-Hz model—Mouse.

Allopregnanolone (3α-hydroxy-5α-pregnan-20-one; 5α,3α-P) is an endogenous progesterone-derived steroid implicated in the regulation of seizure susceptibility in various clinical conditions, most notably catamenial epilepsy (1). 5α,3α-P and certain analogues protect against seizures in diverse animal models, including those induced by γ-aminobutyric acid $(GABA)_A$-receptor antagonists such as pentylenetetrazol (PTZ) (2–5). The potencies of 5α,3α-P analogues in the PTZ model vary largely in accordance with their activities as positive allosteric modulators of $GABA_A$ receptors. Neuroactive steroids are inactive or only weakly active against electrically induced seizures elicited according to the maximal electroshock (MES) protocol that is widely used for antiepileptic drug (AED) screening (60-Hz stimulation for 0.2 s) (3,6). Moreover, the structural specificity of steroids in the MES test does not correspond with their activities as $GABA_A$-receptor modulators, indicating that they act by a distinct mechanism (6).

In recent years there has been a resurgence of interest in an alternative electroshock model in which a 6-Hz electrical stimulus is administered for a prolonged period (3 s). Originally described and pharmacologically characterized by Brown et al. (7), the 6-Hz model appears to be sensitive to a different spectrum of AEDs than is the MES test and may detect agents that are ineffective in standard AED screening models (8). Interestingly, although benzodiazepines are very weakly active in the MES test (>500-fold less potent than in the PTZ model), they are

Accepted March 20, 2004.
Address correspondence and reprint requests to Dr. M.A. Rogawski at Epilepsy Research Section, NINDS, NIH, Building 49, Room 5A75, 49 Convent Drive MSC 4457, Bethesda, MD 20892-4457, U.S.A. E-mail: michael.rogawski@nih.gov

Present address of Mr. Livingood: Duke University, Box 98715, Durham, NC 27708-1627, U.S.A.

highly effective in the 6-Hz model, with potencies equivalent to those in the PTZ test (8). We therefore reasoned that neuroactive steroids might similarly be protective against 6-Hz seizures. In the present study, we report that 5α,3α-P and several analogues with $GABA_A$-receptor potentiating activity are effective in the 6-Hz model with potencies similar to those in the PTZ model.

MATERIALS AND METHODS

Animals

Male NIH Swiss mice, weighing 25 to 30 g, were housed five per cage with free access to food and water. Mice were kept in a vivarium under controlled laboratory conditions (temperature, 22–26°C, humidity 40–50%) with an artificial 12-h light/dark cycle. All animals were allowed to acclimate for ≥5 days before testing. The experimental groups consisted of six to eight animals. The experiments were performed during the light cycle after ≥30-min acclimation to the experimental room. Animals used in these studies were maintained in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and were performed under protocols approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke in strict compliance with the Guide for the Care and Use of Laboratory Animals of the National Research Council (National Academy Press, Washington, DC; http://www.nap.edu/readingroom/books/labrats/).

6-Hz model

The 6-Hz model was carried out according to the protocol originally described by Brown et al. (7) and more recently by Barton et al. (8). Corneal stimulation (0.2 ms–duration monopolar rectangular pulses at 6 Hz for 3 s) was delivered by a constant-current device (ECT Unit 5780; Ugo Basile, Comerio, Italy). A fixed current intensity of 32 mA was used to allow direct comparison with the data obtained in the aforementioned studies. Ocular anesthetic (0.5% tetracaine) was applied to the corneas 15 min before stimulation, and just before stimulation, the corneal electrodes were wetted with 0.9% saline. During the stimulation, mice were manually restrained and released into the observation cage (28 × 20 × 15 cm) immediately after the current application. The seizures were often preceded by a brief period (~2–3 s) of intense locomotor agitation (wild running and jumping). The animals then exhibited a "stunned" posture associated with rearing (bipedal standing), forelimb automatic movements and clonus, twitching of the vibrissae, and Straub-tail. The duration of the seizure activity ranged from 60 to 120 s in untreated animals. At the end of the seizure, animals resumed their normal exploratory behavior. The experimental end point was protection against the seizure. The animal was considered to be protected if it resumed its normal exploratory behavior within 10 s from the stimulation. For CC_{50} (stimulation current causing seizures in 50% of animals) determination, different current intensities in the range of 8 to 32 mA were administered to separate groups of animals.

Data analysis

The CC_{50} value and 95% confidence interval were determined by a logistic fit to the quantal percentage response data. ED_{50} values (dose protecting 50% of animals) and their 95% confidence intervals were similarly determined by nonlinear curve fitting of the percentage protection data at different doses constrained between 0 and 100% protection.

Drugs

5α,3α-P (3α-hydroxy-5α-pregnan-20-one), 5β,3α-P (3α-hydroxy-5β-pregnan-20-one), 5α,3β-P (3β-hydroxy-5α-pregnan-20-one), and CZP [5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2*H*-1,4-benzodiazepin-2-one] were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Ganaxolone (3α-hydroxy-3β-methyl-5α-pregnan-20-one; CCD 1042) was obtained from CoCensys Inc. (Irvine, CA, U.S.A.). Stock solutions of all the drugs were dissolved with mild warming and sonication in a 40% wt/vol solution of hydroxypropyl-β-cyclodextrin (CDT Inc., High Springs, FL, U.S.A.) in saline. Steroids were administered intraperitoneally (i.p.) at doses of 3–100 mg/kg in an injection volume of 0.01 ml/g body weight. Testing was carried out 15 min after the injection, which has been previously shown to be the time of peak anticonvulsant activity (3,5). CZP (0.025–0.2 mg/kg) was administered i.p. 30 min before testing, as described by White et al. (9).

RESULTS

Corneal stimulation with currents in the range of 8 to 32 mA resulted in a current-dependent increase in the fraction of mice reaching the designated seizure end point (Fig. 1). The CC_{50} value determined from the data presented in Fig. 1 is 10.3 (95% confidence limits, 8.9–11.8) mA. The pharmacologic studies were carried out with 32-mA stimulation, which is 3.1 times the CC_{50} value and always resulted in seizure behavior.

Pretreatment with ganaxolone, 5α,3α-P, or 5β,3α-P produced dose-dependent protection against 6-Hz seizures (Fig. 2). The ED_{50} values derived from the experiment of Fig. 2 are presented in Table 1. 5α,3β-P did not show protective activity at a dose of 100 mg/kg. The benzodiazepine CZP, like the neuroactive steroids, afforded dose-dependent protection, but was much more potent.

DISCUSSION

The 6-Hz model originally described by Brown et al. (7) was recently reevaluated by Barton et al. (8). Although the seizure end point was essentially identical in both studies, some differences were found in the CC_{50} values obtained in the two studies. Brown et al. reported a value of 8 mA,

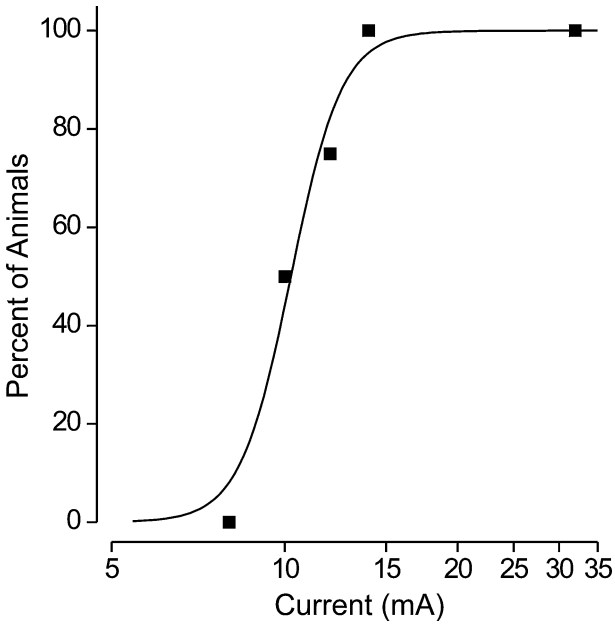

FIG. 1. Incidence of seizures at different 6-Hz stimulation intensities for determination of CC_{50} value. Data points indicate percentage of animals exhibiting seizures at the stimulation current level. Each point represents six to eight animals.

whereas Barton et al. obtained a value of 19.4 mA. The CC_{50} value established in the present study (10.3 mA) was close to that obtained by Brown et al. In both prior studies, a fixed current intensity of 32 mA was used for AED evaluation, although this value is 4 and 1.65 times the respective CC_{50} values. To obtain data comparable to those of the prior studies, we also used a current intensity of 32 mA. Barton et al. found that seizures induced by higher stimulation intensities were more resistant to drugs, and some agents became inactive at these higher intensities.

TABLE 1. *Pharmacologic profiles of neuroactive steroids and clonazepam in the 6-Hz, pentylenetetrazol, and maximal electroshock seizure models in mice*

Test compound	6-Hz test (ED_{50})	PTZ test (ED_{50})	MES test (ED_{50})
Ganaxolone	6.3 (4.0–9.8)	3.5[a] (2.1–5.8)	29.7[b] (25.3–34.8)
5α,3α-P	14.2 (10.3–19.4)	13.7[c] (10.1–18.7)	>100[c]
5β,3α-P	14.4 (10.4–19.9)	18.2[c] (14.7–22.6)	>100[c]
5α,3β-P	>100	>100[c]	>100[c]
Clonazepam	0.075 (0.04–0.14)	0.044[c] (0.02–0.11)	25.6[d] (9.1–65.9)

ED_{50} values in the 6-Hz test from the present study are shown with literature values in the PTZ and MES tests. Testing was carried out 15 and 30 min after injection for neuroactive steroids and clonazepam, respectively. ED_{50} values (with 95% confidence intervals) represent the dose in milligrams per kilogram that is protective in 50% of animals.
PTZ, pentylenetetrazol; MES, maximal electric shock.
[a]Reddy and Rogawski (15).
[b]Carter et al. (12) (tested at 10 min).
[c]Kokate et al. (3).
[d]White et al. (9).

Therefore the relatively high intensity stimulation we used (3.1 times the CC_{50}) represents a rather stringent test of activity.

Barton et al. (8) reported that CZP, tiagabine, and phenobarbital, which act exclusively or predominantly through enhancement of GABA-mediated inhibition (10), are among the most potent of the broad spectrum of AEDs that exhibit protective activity in the 6-Hz model. This suggests that the model is especially sensitive to drugs that act on GABA systems. The present study provides strong support for this concept. We found that neuroactive steroid analogues with GABA-modulating

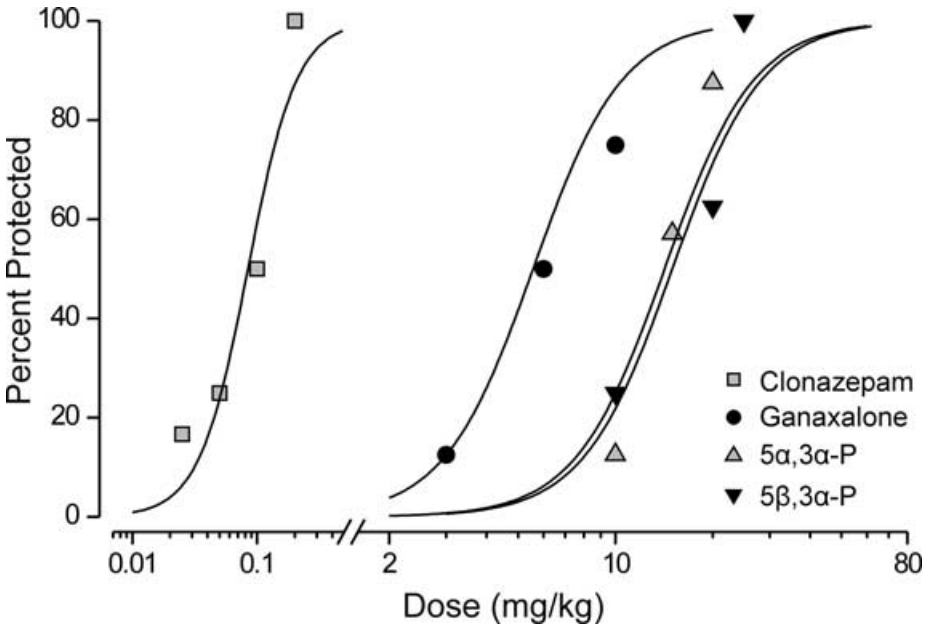

FIG. 2. Protective activities of neuroactive steroids and clonazepam in the 6-Hz model. Data points indicate percentage of animals protected from seizures at the corresponding dose. Each point represents six to eight animals.

activity (ganaxolone; 5α,3α-P; 5β,3α-P) were highly effective in the model, whereas 5α,3β-P, a stereoisomer of 5α,3α-P that has very weak activity on $GABA_A$ receptors (3), was inactive in the seizure model. We also confirmed that CZP, a benzodiazepine that potently potentiates $GABA_A$-receptor currents (3,11), is highly effective in the model. Although the rank order of potencies of the steroids in the 6-Hz model largely corresponds with their potencies as $GABA_A$-receptor modulators, a discrepancy occurs in the case of ganaxolone. In the in vitro studies, 5α,3α-P and ganaxolone influence $GABA_A$-receptor activity with similar potencies, potentiating GABA-evoked Cl^- currents at low concentrations and directly activating the $GABA_A$ receptor at higher concentrations (12). The greater potency of ganaxolone in the 6-Hz and PTZ models (Table 1) probably reflects the enhanced bioavailability of ganaxolone (13).

Interestingly, Barton et al. (8) found that AEDs effective in the 6-Hz model (CZP, phenobarbital, valproate, ethosuximide, tiagabine) also are effective in the PTZ model and have similar potencies. These drugs are weaker or ineffective in the MES test (9). In contrast, drugs effective in the MES test (phenytoin, carbamazepine, lamotrigine, felbamate, topiramate) are weak or ineffective in both the 6-Hz and PTZ models (8). The active 5α,3α-P analogues examined in the present study belong in the former category: they are highly effective in the PTZ model (3,6), but are ineffective (5α,3α-pregnanolone and 5β,3α-pregnanolone; ref. 3) or effective only at toxic doses (ganaxolone; ref. 12) in the MES test (Table 1). As such, their pharmacologic profile resembles the profiles of AEDs that are effective in the 6-Hz model, and we found that the active steroids are highly effective in the 6-Hz model. The protective effects of 5α,3α-P analogs in the 6-Hz model exhibit the same stereoselectivity as in the PTZ test (3). This same structural specificity also applies to other seizure models, including the pilocarpine and kainic acid models (14). The PTZ test is well recognized to be sensitive to drugs that act on $GABA_A$ receptor–mediated inhibition (10), further confirming the proposed mechanism.

In the present study, we have shown that neuroactive steroids related to 5α,3α-P are protective in the 6-Hz model. Protection occurred in a dose-dependent fashion, with structural specificity that corresponds with the activity of the steroids in the PTZ test, consistent with the possibility that $GABA_A$ receptors represent the target in this model. We also confirmed that CZP shows high potency in the model. Taken together, these findings indicate that the 6-Hz model is highly sensitive to AEDs that positively modulate $GABA_A$ receptors and thus may be a valuable tool in the identification of such compounds.

REFERENCES

1. Rogawski MA, Reddy DS. Neurosteroids: endogenous modulators of seizure susceptibility. In: Rho JM, Sankar R, Cavazos J, eds. *Epilepsy: scientific foundations of clinical practice*. New York: Marcel Dekker, 2004:319–55.
2. Belelli D, Bolger MB, Gee KW. Anticonvulsant profile of the progesterone metabolite 5α-pregnan-3α-ol-20-one. *Eur J Pharmacol* 1989;166:325–9.
3. Kokate TG, Svensson BE, Rogawski MA. Anticonvulsant activity of neurosteroids: correlation with γ-aminobutyric acid-evoked chloride current potentiation. *J Pharmacol Exp Ther* 1994;270:1223–9.
4. Wieland S, Belluzzi JD, Stein L, et al. Comparative behavioral characterization of the neuroactive steroids 3α-OH,5α-pregnan-20-one and 3α-OH,5β-pregnan-20-one in rodents. *Psychopharmacology* 1995;118:65–71.
5. Gasior M, Carter RB, Goldberg SR, et al. Anticonvulsant and behavioral effects of neuroactive steroids alone and in conjunction with diazepam. *J Pharmacol Exp Ther* 1997;282:543–53.
6. Kokate TG, Banks MK, Magee T, et al. Finasteride, a 5α-reductase inhibitor, blocks the anticonvulsant activity of progesterone in mice. *J Pharmacol Exp Ther* 1999;288:679–84.
7. Brown WC, Schiffman DO, Swinyard EA, et al. Comparative assay of antiepileptic drugs by "psychomotor" seizure test and minimal electroshock threshold test. *J Pharmacol Exp Ther* 1953;107:273–83.
8. Barton ME, Klein BD, Wolf HH, et al. Pharmacological characterization of the 6 Hz psychomotor seizure model of partial epilepsy. *Epilepsy Res* 2001;47:217–27.
9. White HS, Woodhead JH, Wilcox KS, et al. General principles: discovery and preclinical development of antiepileptic drugs. In: Levy RH, Mattson RH, Meldrum BS, et al., eds. *Antiepileptic drugs*. 5th ed. Philadelphia: Lippincott Williams & Wilkins, 2002:36–48.
10. Rogawski MA. Principles of antiepileptic drug action. In: Levy RH, Mattson RH, Meldrum BS, et al., eds. *Antiepileptic drugs*. 5th ed. Philadelphia: Lippincott Williams & Wilkins, 2002:3–22.
11. Korpi ER, Grunder G, Luddens H. Drug interactions at $GABA_A$ receptors. *Prog Neurobiol* 2002;67:113–59.
12. Carter RB, Wood PL, Wieland S, et al. Characterization of the anticonvulsant properties of ganaxolone (CCD 1042; 3α-hydroxy-3β-methyl-5α-pregnan-20-one), a selective, high-affinity, steroid modulator of the γ-aminobutyric $acid_A$ receptor. *J Pharmacol Exp Ther* 1997;280:1284–95.
13. Monaghan EP, McAuley JW, Data JL. Ganaxolone: a novel positive allosteric modulator of the $GABA_A$ receptor complex for the treatment of epilepsy. *Expert Opin Invest Drug* 1999;8:1663–71.
14. Kokate TG, Cohen AL, Karp E, et al. Neuroactive steroids protect against pilocarpine- and kainic acid-induced limbic seizures and status epilepticus in mice. *Neuropharmacology* 1996;35:1049–56.
15. Reddy DS, Rogawski MA. Enhanced anticonvulsant activity of ganaxolone after neurosteroid withdrawal in a rat model of catamenial epilepsy. *J Pharmacol Exp Ther* 2000;294:909–15.